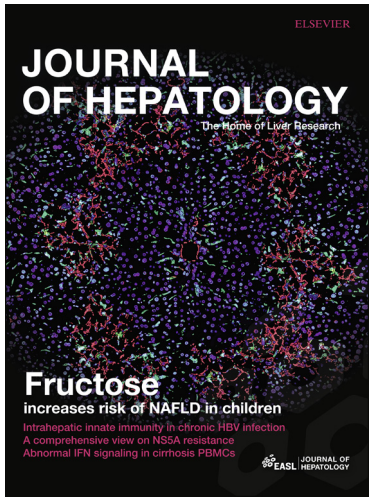

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Identification of a xenobiotic as a potential environmental trigger in primary biliary cholangitis

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Abbreviations: AhR, aryl hydrocarbon receptor; AMA, anti-mitochondrial antibody; AR, androgen receptor; β-NF, β-naphthoflavone; COOH7IM, 1-(7-carboxyheptyl)-3-methyl-1H-imidazol-3-ium; ERα, estrogen receptor alpha; LT, lipoyl-AMP(GMP):N-lysine lipoyl transferase; LAE, lipoate activating enzyme; M8OI, 3-methyl-1-octyl-1H-imidazol-3-ium; 3MC, 3 methylcholanthrene; PAH, polyaromatic hydrocarbon; PBC, primary biliary cholangitis; PBS, phosphate buffered saline; PDC-E2, E2 component of the pyruvate dehydrogenase complex, also known as dihidrolipoamide-S-acetyl transferase (DLAT); PPARα, peroxisome proliferator activating receptor; PSC, primary sclerosing cholangitis.

Background and Aims: Primary biliary cholangitis (PBC) is an autoimmune-associated chronic liver disease triggered by environmental factors - such as exposure to xenobiotics - leading to a loss of tolerance to the lipoic acid conjugated regions of the mitochondrial branched-chain α-ketoacid dehydrogenase complex, typically to the E2 component (PDC-E2). **Methods:** Urban landfill and control soil samples from a region with high PBC incidence were screened for xenobiotic activities using analytical, cell-based xenobiotic receptor activation assays and toxicity screens. **Results:** A variety of potential xenobiotic classes were ubiquitously present, as identified by their interaction with xenobiotic receptors - aryl hydrocarbon (AhR), androgen (AR) and peroxisome proliferator activated receptor alpha (PPARα) receptors - in cell-based screens. In contrast, xenoestrogen – estrogen receptor (ERα) - interacting chemicals were present at higher levels in soil extracts from around an urban landfill. Furthermore, two landfill sampling sites contained a chemical(s) that inhibited mitochondrial oxidative phosphorylation and induced the apoptosis of an hepatic progenitor cell. The mitochondrial effect was also demonstrated in human liver cholangiocytes from 3 separate donors. The chemical was identified as the ionic liquid [3-methyl-1-octyl-1*H*-imidazol-3-ium]$^+$ (M8OI) and the toxic effects were recapitulated using authentic pure chemical. A carboxylate-containing human hepatocyte metabolite of M8OI - bearing structural similarity to lipoic acid - was also enzymatically incorporated into the E2 component of pyruvate dehydrogenase via the exogenous lipoylation pathway in vitro. **Conclusions:** These results identify for the first time, a xenobiotic in the environment that may be related to and/or potentially be a component of an environmental trigger for PBC.

Abstract word count: 252

Lay summary: PBC is a liver disease in which most patients have antibodies to mitochondrial proteins containing lipoic acid binding site(s). This paper identified a man-made chemical present in soils around

a waste site and shows that it is metabolised to a product having structural similarity to lipoic acid and is capable of replacing lipoic acid in mitochondrial proteins.

Liver disease constitutes the third commonest cause of premature death in the UK with an upward trend in mortality in the UK[1,2]. The major causes of liver disease such as obesity, viral infection and chronic alcohol consumption are preventable. However, the causes of rarer types of liver disease – such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are unknown and as a consequence, prevention and/or treatments are limited[3]. A variety of factors have been linked to increased incidence of PBC and PSC in populations. In both cases, although there is a clear genetic predisposition[4,5], there is also evidence that environmental factors – such as exposure to foreign compounds (xenobiotics) – determine the likelihood of developing disease[6-9].

In the majority of cases, PBC is associated with an immunological loss of tolerance to the lipoic acid-conjugated regions of the mitochondrial branched chain α keto acid dehydrogenase complex, leading to the presence of high levels of diagnostic anti-mitochondrial antibodies (AMA) in patient sera[10,11]. Thus, one potential trigger for PBC may be exposure to chemicals that structurally and chemically mimic lipoic acid and may therefore be capable of enzymatic incorporated into PDC-E2 in place of lipoic acid[10,11]. Specific disease pathology in the liver (since the antigen for AMA is present in most cells of the body) is thought to be due to selective exposure of the neo-antigen (e.g. via selective apoptosis of hepatic intrahepatic duct cells/cholangiocytes)[12].

A number of signalling pathways have been identified in cells that function to detect xenobiotics and modulate gene expression in order to facilitate their metabolism and excretion. Polycyclic aromatic hydrocarbons (PAHs) are environmental organic xenobiotics consisting of 2 or more clustered benzene rings and are present in crude oil and coal tar and contaminate the environment through fossil fuel burning, incineration of waste and other industrial processes[13]. They are hydrophobic and often persist in the environment and tissues. Many PAHs are suspected or known to be toxic, genotoxic and carcinogenic[14]. The aryl hydrocarbon receptor (AhR) binds and is transcriptionally activated by many PAHs[15]. In the liver, this leads to the increased expression of a variety of genes associated with xenobiotic metabolism (e.g. the Ah locus)[15]. However, the function of the AhR likely extends beyond

xenobiotic metabolism alone as its endogenous functions appear to include roles in the immune system[16]. In the thymus, AhR activation can lead to thymocyte apoptosis and thymic atrophy[17]. The peroxisome proliferator activated receptor alpha (PPARα) is a nuclear receptor that functions in the regulation of fatty acid oxidation. In addition to its activation by selected endogenous lipids, the receptor is also activated by fibrate drugs (its pharmacological target) and xenobiotics such as polyhalogenated chemicals e.g. perfluorooctane sulfonate[18]. The nuclear estrogen receptor alpha (ERα) appears to be a frequent target for a variety of natural (e.g. plant phytoestrogens) and xenobiotic man-made chemicals (e.g. pesticides). Xenoestrogens have been proposed to be responsible for a spectrum of adverse effects in wildlife and man that include malformations in the male genital tract; decreased sperm quality; neuroendocrinological, behavioural and metabolic effects and cancer[19-21].

In an attempt to identify potential environmental xenobiotic triggers, urban landfill and control soil samples from a region with high PBC incidence were screened for xenobiotic activities using a variety of in vitro cell-based assays.

MATERIALS AND MTHODS

Chemicals

3-methyl-1-octyl-1H-imidazol-3-ium **(**M8OI) was purchased from Sigma (Poole, UK). 1-(8-hydroxyoctyl)-3-methyl-imidazolium (HO8IM) and 1-(7-carboxyheptyl)-3-methyl-1H-imidazol-3-ium (COOH7IM) were custom synthesized with purity and chemical structures determined by HPLC, mass spectrometry and NMR techniques (for COOH7IM, see **Supplementary Fig. 11**).

Preparation of soil extracts

Surface soil samples (0-5cm in depth) were collected and extraneous vegetable matter and stones removed. Each sample was divided into four 250g portions. A sample of one portion was digested using *aqua regia* in accordance with BS7755 for metals analysis. Two portions were subjected to either

methanol (for polar molecule) or chloroform (for hydrophobic chemical) extractions by sonicating with 300mls of solvent for 10 mins, followed by addition of a further 100mls of solvent and sonication for a further 10 mins prior to filtration with 25µm filters and collection of filtrate. Filtrates were evaporated in a rotary evaporator and then blown down to near dryness under a stream of nitrogen. The methanol extracted material was divided into two and added to either 10mls of phosphate buffered saline (PBS, 137 mM NaCl, 27 mM KCl, 100 mM phosphate pH 7.4) or 10mls ethanol. The chloroform extracted material was re-dissolved into 10mls chloroform. The solvated extracted chemicals were then separated from any precipitate and stored at -20^{o}C (ethanol and chloroform extracts) or 4^{o}C (PBS extracts).

Thirteen soil samples were collected from allotments, footpaths and the roadside verges surrounding an urban landfill site. Three control soil samples were collected from 3 separate sites. One sample was obtained from the University farm in rural Northumberland at a site with controlled fertiliser regime for the last 130 years. The remaining 2 control samples were obtained from gardens in urban areas in the region.

Cell culture

Rat B-13 hepatocyte progenitor cells were routinely expanded in low glucose (1000mg/l) Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) FCS, 80u/ml penicillin and 80µg/ml streptomycin. B-13 cells were converted into functional hepatocytes (B-13/H cells) in vitro through addition of 10nM dexamethasone essentially as previously outlined[52,53,23]. B-13/H cells are a non-proliferative functional hepatocyte-like cell expressing a variety hepatic functions (such as functional cytochrome P450s) at near normal liver levels[54]. The human H69 cholangiocyte cell line[55] was routinely expanded in 3:1 (v/v) ratio of DMEM and Nutrient F12 Ham's medium supplemented with 180µM adenine, 2nM triiodothyronine, 5.5µM epinephrine, 1µM hydrocortisone, 10% v/v FCS, 1x Insulin/transferrin/selenium (Gibco) and 1 x Pen/Strep (Lonza). The human hepatoma HepG2 cell lines was cultured as previously described[56]. The human breast cancer MCF-7 cell line was cultured as

previously described[57]. All cells were incubated at 37°C in an humidified incubator gassed with 5% CO_2 in air. Human cholangiocytes were isolated from resected human liver using an immune-bead approach as previously described and cultured in 1:1 [v/v] DMEM:Hams F12 medium supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF), 0.248 IU/ml Insulin, 2 µg/ml hydrocortisone, 10 ng/ml cholera toxin, 2nM tri-iodo-L-thyronine and 5 ng/ml hepatocyte growth factor (HGF)[58]. Human hepatocytes were isolated from a 42 year old male donor by collagenase perfusion essentially as previously described[59] and cultured on collagen-coated plates in Williams medium E supplemented with 10% (v/v) FCS, 80u/ml penicillin, 80µg/ml streptomycin, 10nM dexamethasone and 1ug/ml insulin. After an overnight culture period, the medium was aspirated, the cells were washed 3 times with sterile PBS prior to incubation with M8OI in a short-term simplified incubation medium (STIM buffer: 0.10M NaCl, 5.4mM KCl, 0.34mM Na_2HPO_4 $12H_2O$, 0.44mM KH2PO4, 20mM glucose, 1mM $CaCl_2$, 40mM $NaHCO_3$, 4mM glutamine, 100µM L-alanine, 100µM L-asparagine, 100µM L-aspartic acid, 100µM L-glutamic Acid, 100µM glycine, 100µM L-proline and 100µM L-serine, pH 7.4 when gassed with 5% CO_2 in air) to minimise interference with M8OI detection, typically 1.5mls/well of a 6 well plate. As a control, M8OI was incubated identically in a cell-free culture vessel. After 24 hours, the STIM incubation was removed, centrifuged at 13,000 rpm for 1 min and 10 volumes of supernatant clear of any cellular material retained and added to 1 volume 1% phosphoric acid. Acid-precipitated material was removed by centrifugation (13,000 rpm, 1min) and the supernatant was retained at stored at 4°C prior to analysis. Human tissue was obtained with patient consent and with approval of the Newcastle & North Tyneside 2 Research Ethics Committee.

Animal study

A study with mice detailed in the supplementary data section was performed under a UK Home Office license with Local Ethics Committee approval. Animals (up to 5 per cage) were housed in Maxiseal 420 cm^2 mouse cages (Arrowmight, Hereford, UK) in an enriched environment (nesting material, chew sticks

and cardboard tubes) and were provided with food (RM3 Special Diet Services, UK) and water ad libitum in an air-conditioned environment on a 12 hour light/dark cycle with regulated humidity (50% ± 10%) and temperature (23°C ± 1°C). All animals received humane care and the study was in compliance with institutional and ARRIVE guidelines. For additional information, see Supplementary data (includes references 52-67).

RESULTS

Similar levels of heavy metals, polyaromatic hydrocarbons and pesticides were present in landfill and control site soils.

Soil samples were taken from around a currently active peri-urban landfill site (and from 3 separate control sampling sites), with all sites positioned upon an area of historic coal mining. Extracts obtained using different solvents – from aqueous to hydrophobic - were prepared and tested as schematically outlined in **Supplementary Fig. 1a**. Analysis of both sonicated soil extracts and the *aqua regia* digests indicated low levels of contamination with a wide range of potentially toxic elements (**Supplementary Table 1**). At all sample sites, values were typical of an urban soil lacking overt contamination[22]. These data were supported by a metallothionein luciferase reporter gene assay screen, which indicated low levels of metals in all soil extracts (**Supplementary Fig. 1b**). Since the landfill and control sites were also situated in a region with a history of intensive coal mining, the soil levels of common PAHs were also examined. **Supplementary Table 2** indicates that levels were not significantly different although overall, the mean levels were higher in control soils (65 ± 54 mg/kg soil) compared to landfill soils (13 ± 19 mg/kg soil). A screen for 24 different pesticides indicated that 22 out of 24 were detectable in at least one soil sample and a minimum of at least 4 different pesticides (landfill soil sample 2) were detectable in each soil sample (**Supplementary Table 3**). In all samples, the levels were low with only 2 pesticides - Diuron and Omethoate – present at levels greater than 1ppm in any soil extract (the former, at mean higher levels in control soils compared to soils in close proximity to the landfill site). The combined mean

pesticide levels were higher in control soils (total of 3.9 +/- 1.81 ppm) compared to soils in close proximity to the landfill site (total of 2.0 +/- 1.69 ppm).

To screen for the presence of acutely toxic chemicals, extracts were screened for toxicity in hepatic progenitor B-13, hepatocyte-like B-13/H and H69 cholangiocyte cell lines[23] at an initial concentration of 1% (v/v) in culture media. **Supplementary Fig. 2** demonstrates that – with the exception of PBS solvated extracts from 2 landfill sampling sites and B-13 cells – none of the extracts were markedly toxic. To reduce the potential that toxic effects might interfere with interaction with signalling pathways, a further 10 fold dilution of soil extracts were routinely examined (i.e. 0.1% (v/v) in culture media). **Supplementary Fig. 3a** demonstrates that extracts from both the landfill sampling sites and control sites induced several hundred-fold increases in AhR-dependent XRE-luc reporter gene activity, with higher fold induction observed in extracts solvating more hydrophobic chemicals (i.e. chloroform extracts). These data were confirmed by similar robust induction in AhR-regulated Cyp1a1 mRNA expression in metabolically-active xenobiotic metabolising B-13/H cells (**Supplementary Fig. 3b**); a dose-response effect in all selected tested extracts (**Supplementary Fig. 3c**) and induction of Cyp1a1 protein expression (**Supplementary Fig. 3d**). Similar results were also found when ethanol and chloroform extracts were examined for activators of the human PPARα (**Supplementary Fig. 4**), demonstrating that soil extracts from both landfill and control sites contain chemicals that activate xenobiotic receptor AhR and PPARα receptors.

Chemicals present in landfill site soils activated the human ERα, with limited effects on the human AR.

To determine whether soil extracts contained xenoestrogens capable of activating the human ERα, MCF-7 cells transfected with an estrogen responsive luciferase reporter gene (ERE_3-pGL3promoter), were exposed to soil extracts. PBS and chloroform extracts were not toxic (**Supplementary Fig. 5a,b**) and in a minority of some landfill soil samples, contained chemicals that activated the human ERα

(**Supplementary Fig. 5d,e**). However, ethanol soil extracts – which were also not toxic to MCF-7 cells (**Fig. 1a**), see also **Supplementary Fig. 5c**) - contained the most potent ERα activation activities, with activation restricted to several soil samples in close proximity to a landfill site (**Fig. 1b**). There was low to undetectable estrogenic activity detectable in ethanol extracts from the control soil samples (**Fig. 1b**). Clear dose-responses were observed through further dilution of ethanol extracts (**Fig. 1c**). Inhibition of activation by co-incubation with the anti-estrogen ICI182780 (**Fig. 1d**) confirmed direct ERα interaction and activation of transcriptional function by the ethanol extracts from soil samples in proximity to a landfill site. In contrast, there was limited activity directed toward the human AR with only control site 2 yielding a statistically significant increase in reporter gene expression likely not to be of biological significance (**Fig. 1e**). Two landfill soil sites contained chemicals capable of antagonising DHT activation of the human AR (**Fig. 1f**).

These data demonstrate that – in contrast to AhR and PPARα-activating chemicals - several extracts from soil samples taken from sites in close proximity to a landfill site contain human ERα-activating chemicals, in contrast to control soil samples.

Two aqueous landfill soil extracts blocked hepatic progenitor cell proliferation and induced cell death.

PBS extracts from 2 sampling sites in close proximity to the landfill site showed marked toxicity in the hepatic B-13 progenitor cell line (**Supplementary Fig. 2a**) – with less sensitivity in the xenobiotic metabolically-active B-13/H hepatocyte cell derived therefrom (**Supplementary Fig. 2d**). Since there was a lack of apparent sensitivity in H69 (**Supplementary Fig. 2g**) and MCF-7 cells (**Supplementary Fig. 5a**), the nature of this toxicity was examined in the B-13 liver progenitor cell. The earliest observed effects of PBS 1 and 2 extracts on B-13 cells was an inhibition in proliferation, which was confirmed by a marked inhibition of DNA synthesis (**Fig. 2a**), an initial inhibition in cell numbers followed by cell death between 24 and 48 hours after exposure (**Fig. 2b**). **Fig. 2c** demonstrates a dose-response effect of the

toxic PBS extracts on B-13 MTT reduction. Since the cells excluded trypan blue subsequent to effects on proliferation and MTT reduction, it was hypothesised that B-13 cells undergo an apoptotic mechanism of cell death. PBS extracts 1 and 2 induced a marked increase in caspase 3/7 activity (**Fig. 2d**) and cleavage of genomic DNA to generate classic nucleosomal ladders (**Fig. 2e**), supporting an initial apoptotic mode of cell death.

To further characterise the causative mechanism(s) inducing B-13 cell apoptosis, the potential role of mitochondria was examined since this organelle plays a critical role in apoptotic mechanisms of cell death. Replacing media glucose with galactose has been reported to increase cellular susceptibility to mitochondrial toxicants[24]. **Fig. 3a** demonstrates that replacing glucose with galactose sensitised B-13 cells – in a dose responsive manner – to the toxic effects of the toxic PBS extracts. Measurements of oxygen consumption rates in B-13 cells using a seahorse analyser confirm that the toxic PBS extracts inhibited oxidative phosphorylation, targeting in particular maximal respiration and ATP production (**Fig. 3b,c**). B-13/H mitochondria were less sensitive to the toxic PBS extracts, experiencing weaker inhibitory effects on both maximal respiration rate and ATP production (see **Supplementary Fig. 8a**), supporting a role for the mitochondrial effects of toxic PBS extracts in B-13 cell death. **Fig. 3d** demonstrates that toxic PBS extract exposure resulted in a dose-dependent decrease on oxygen consumption rate (OCR) and a dose-dependent increase in extracellular acidification rate (ECAR) in B-13 cells. The increase in ECAR was considered likely due to an inhibition of mitochondrial oxidative phosphorylation and compensatory regulated changes in metabolism such as glycolytic flux. This hypothesis was supported by decreases in cellular ATP in cells treated with toxic PBS extracts when glucose was limited (**Fig. 3e**); a protection from toxicity through increasing glucose concentration (data not included) and an activation of AMP activated protein kinase (AMPK) by phosphorylation, an effect not observed when cells were treated in high glucose media (**Fig. 3f**).

These data indicate that the PBS extracts from 2 sampling sites in close proximity to the landfill site contain a chemical(s) that inhibited mitochondrial oxidative phosphorylation.

The landfill toxic PBS extracts inhibit cultured human cholangiocyte mitochondrial function

To establish whether the toxic response of B-13 cells to toxic PBS extracts could be relevant to man, cholangiocytes were isolated from resected human liver and exposed to toxic PBS extracts or control PBS although limitations in tissue and cell numbers precluded extensive analyses. **Fig. 4a** demonstrates that the cells isolated from 3 donors expressed the biliary marker cytokeratin 19 and therefore that an enriched population of cholangiocytes were isolated and cultured. Application of the seahorse approach to determine effects on OCR demonstrated that both toxic PBS extracts inhibited mitochondrial function in a similar manner to that observed in B-13 cells (**Fig. 4b**). Maximal respiration and ATP production was also affected by the PBS extracts as observed with B-13 cells but in addition, basal respiration rates were also inhibited (**Fig. 4c**).

These limited analyses in human cholangiocytes therefore suggest that the adverse effects of the toxic PBS extracts could be relevant in man if the ductular regions of the liver are exposed to sufficient concentrations of the chemical(s).

The toxic chemical present landfill aqueous soil extracts was the ionic liquid 3-methyl-1-octyl-1H-imidazol-3-ium^{+}

PBS extracts from landfill sampling sites 1 and 2, were subjected to TLC and spectrophotometry and determined to be a non-fluorescent chemical(s) lacking extensive bond conjugation (**Supplementary Figure 6a,b,c**). The presence of bacterial agents and/or toxic proteins were also excluded (**Supplementary Figure 6d,e**). Exposing B-13 cells to separate HPLC fractions determined that the toxic effects associated with the extracts co-eluted with the major peak detected, not detectable in the control and non-toxic landfill PBS extracts after identical batch fraction collection (**Fig. 5a**). Mass spectrometric analyses of the material eluting with this peak suggested the presence of a single species corresponding to a singly charged [M+H] of 195.1861 Da (**Fig. 5b**). Interrogation of databases predicted the best elemental

fit to the parent ion of $C_{12}H_{22}N_2$, with a mass error of 5ppm. The isotope pattern did not suggest the presence of chlorine or sulphur in the molecule. Mass spectrometry and fragmentation analyses suggested that the chemical consists of 2 alkyl chains and a cyclic moiety containing the two nitrogen atoms (**Fig. 5b**). However, the predicted molecular formula could, in theory, be generated by 3316 potential distinct chemical structures according to Chemspider (http://www.chemspider.com/). Repeated fractions containing this peak were therefore collected, pooled and subjected to NMR analysis (**Supplementary Fig. 7**), identifying the chemical as the ionic solvent 3-methyl-1-octyl-1H-imidazol-3-ium$^+$ (M8OI), see **Fig. 5c** for structure. Exposing B-13 cells to the authentic commercially-available chloride salt (M8OI-Cl) essentially replicated the toxicity of PBS 1 and 2 extracts in terms of effects on mitochondrial function (**Fig. 6a**) including a clear dose-response effect on maximal respiration and ATP production (**Fig. 6b**), ATP levels (**Fig. 6c**), caspase 3/7 activities (**Fig. 6d**), MTT reduction (**Supplementary Fig10a**) and induction of DNA nucleosomal laddering (**Fig. 6e**).

An approximate $EC_{50\%}$ for MTT of 0.2% (v/v) for landfill PBS extract 2 (**Fig. 2c**) and 50µM M8OI for B-13 cells (**Supplementary Fig. 8b**), suggests that the concentration of M8OI in the PBS extract 2 was approximately 25mM.

B-13 cells also showed an increased sensitivity to M8OI compared to the epithelial B-13/H cell in terms of mitochondrial sensitivity (**Supplementary Fig. 8a**), caspase 3/7 induction and loss of MTT reduction activity (**Supplementary Fig. 8b**) and the minimum M8OI concentration inducing a detectable DNA ladder (**Supplementary Fig. 8c**). M8OI was mildly toxic to the human cholangiocyte-like H69 cell line (**Supplementary Fig. 9**) based on MTT reduction assays ($EC_{50\%}$ ~ 500µM, compared ~ 100µM for B-13 cells).

These data indicate that soil samples surrounding a landfill waste site contained the ionic liquid M8OI and that this chemical is toxic to liver progenitor cells through an interaction with their mitochondria, resulting in an induction of apoptosis.

M8OI is metabolised to a carboxylate-containing metabolite by human hepatocytes and is capable of being enzymatically incorporated into the E2 component of pyruvate dehydrogenase

Lipoic acid (for structure, see **Fig. 7d**) is a small endogenously synthesised co-factor that is covalently bound to a variety of enzymes, including the E2 component of pyruvate dehydrogenase complex (PDC-E2). This co-factor is essential for the acyl or methylamine transfer functions of the enzymes to which it is linked[10,11]. Lipoic acid may be synthesised *de novo* from octanoic acid produced during fatty acid synthesis in bacteria. There is also a scavenging pathway that links ATP or GTP hydrolysis to its activation and conjugation to proteins[25] (see **Fig. 7d**).

Although M8OI is persistent it has been shown to be broken down in the environment through oxidation of the terminal carbon (C_{13}) to form the carboxylic acid - 1-(7-carboxyheptyl)-3-methyl-1H-imidazol-3-ium (COOH7IM)[26]. This pathway of catabolism by soil bacteria mirrors the pathway of fatty acid omega oxidation observed in mammalian liver (see **Supplementary Fig. 10b**). COOH7IM was not acutely toxic to B-13 cells (data not shown) and therefore, it was not directly tracked using our approach. Its presence or not in samples soils cannot therefore be determined with certainty.

To determine whether M8OI is metabolised to COOH7IM, cultured human hepatocytes were exposed to M8OI for 24 hours prior to analysis by TripleTOF 5600 high-resolution quadrupole time-of-flight (TOF) mass spectrometry (Sciex). Mass spectrometric scanning of predicted masses for the parent M8OI, hydroxylated metabolite (HO8IM) and COOH7IM was used for detection and demonstrate that M8OI was metabolised to HO8IM and predominantly COOH7IM as predicted (**Fig. 7a**). Analysis of serum samples isolated from mice orally administered M8OI demonstrated that M8OI was absorbed from the gastrointestinal tract, was systemically available and metabolised in the mouse similarly to man since both HO8IM and COOH7IM where detectable in sera (**Fig. 7b**). Interestingly, M8OI was found to be present in bile at levels > 30-fold the concentrations found in sera **(Fig. 7c**), indicating that a proportion of the chemical is cleared intact via biliary excretion. COOH7IM was synthesised and its enzymatic incorporation into PDC-E2 examined. **Fig. 7e** demonstrates the covalent incorporation of lipoic acid into

an unlipoylated (ULip) fragment of the human PDC-E2 protein (PDC-E2-ILD) as previously described[27]. **Fig. 7e** further demonstrates incorporation of the M8OI derivative possessing a carboxylated group on C_{13} (centre panel, COOH7IM), an effect not observed with M8OI itself nor an M8OI derivative possessing a hydroxyl group on C_{13} (i.e. HO8IM, data not shown). COOH7IM incorporation into Ulip was both GTP- and enzyme (LAE/LT)-dependent (**Fig. 7e**, right panel).

DISCUSSION

This study initially aimed to determine the feasibility of extracting chemicals from soil samples and applying them to a selected suite of in vitro toxicity assays in order to screen for any toxicological effects. The hypothesis was that the approach would detect higher levels of chemicals with potential toxic effects around a landfill site compared to sample sites not in proximity to a landfill site. The presence of chemicals activating the AhR was examined because this receptor is known to be activated by PAHs associated with fossil fuel burning and industrial pollution[28]. This receptor mediates most of the toxic effects of dioxin in animals and man, including chloracne, anorexia, thymic atrophy and cancer[29]. The AhR has also been shown to recognize both xenobiotics and natural compounds (tryptophan metabolites, dietary components and microbiota-derived factors), and is important for maintenance of homeostasis at mucosal surfaces through regulation of type 3 innate lymphoid cells and T helper 17 cell activities[30]. The PPARα is the nuclear receptor drug target for fibrate drugs that mediates reductions in lipid levels through increasing peroxisomal oxidation of fatty acids[31]. The presence of chemicals activating the PPARα was examined because environmental surfactant pollutants (e.g. perfluoroalkyl acids) activate this receptor[18]. However, PPARα agonists have also been shown to be protective against cholestatic liver injury[32] and are currently being considered as therapies for PBC and other cholestatic liver diseases[33]. One conclusion from this study is that it is possible to readily detect chemicals from soil extracts that contact and activate the receptors. The results of our analyses indicate that the chemicals activating the AhR or PPARα were widely present in the soils (either naturally and/or through anthropomorphic activity), including soils not in close proximity to a landfill site. Comparison of the potentially toxic element (PTE) content of these

soils, based on metals and polyaromatic hydrocarbon (PAH) analyses, indicates that the soil samples are consistent with soils from urban areas in NE England, and in most cases are lower than the mean for soils in this region[34].

In contrast, chemicals that activated the ERα were at higher levels in soils around a landfill site compared to control soils. A wide variety of man-made chemicals have been suggested to have endocrine-disrupting chemicals in animals and humans[35,36] and many have been shown to have estrogenic properties in that they mimic the biological effects of endogenous estrogens (xenoestrogens). Therefore, in terms of disposal of normal domestic waste, there may be a potential for xenoestrogens to be at higher levels in the surrounding environment, although whether this is hazardous to the environment and to nearby populations remains to be determined.

Given the marked toxic effect of two PBS extracts from 2 sites in close proximity to a landfill, the mechanism of action and identity(ies) of the chemical(s) responsible were investigated. Less potent effects were observed in B-13/H hepatocytes derived from the B-13 progenitor (perhaps due to degradation to less toxic metabolites in these more active xenobiotic metabolising cells) and a variety of other cells such as human cholangiocytes. A single chemical was identified – the ionic liquid 3-methyl-1-octyl-1H-imidazol-3-ium^{+} (M8OI) in the toxic PBS extracts (which also contained estrogenic activity). Since the pure commercially-available chemical induced the same effects in B-13 cells, it is likely that the M8OI alone was responsible for the effects of the toxic PBS extracts. Retrospective non targeted data independent LC-HR-MS/MS analysis of all PBS extracts confirmed that landfill sites 1 and 2 contained high levels of M8OI (the highest - PBS extract 2, 2.4mg/mL equal to 12mM) in good agreement with the estimation from toxicity data of ~ 25mM. M8OI was present at low levels in all other landfill sampling PBS extracts (range 0.0012 – 0.038 mg/L) but not detected in any control sample sites. Based on these analyses, and assuming that all M8OI partitioned into the PBS extract, this means the highest M8OI soil concentration was 0.48mmoles/kg (equivalent to 94 mg/kg soil).

Ionic liquids are salts with a melting temperature below the boiling point of water, are often liquids at room temperature and are often composed of organic cations and inorganic anions[37]. They have been proposed to be a new generation of "green solvents" because they are organic solvents with relatively low volatility, therefore having many of the advantages of molten salts without requiring high temperatures[37]. The first ionic liquid was identified in the mid-19th century, but the modern era of discovery and development occurred with the generation of 1-butylpyridinium chloride–aluminium chloride mixtures in the 1970s[38,37]. They may currently find use in protection products and herbicidal ionic liquids such as derivatives of phenoxyacids exhibiting a selective herbicidal activity against dicotyledonous plants[39]. According to commercial suppliers, M8OI is used/associated with metal plating, electropolishing, metal reprocessing, phase transfer media, batteries, nanomaterials, industrial solvents, nuclear fuel red waste, enzymatic catalysis, lubricants heat transfer and solar energy conversion. Interrogation of the ECHA database shows that 5 different M8OI salts have been pre-registered, but since no data on their toxicity have been submitted, their production level by any single producer should be less than 100 tonnes/annum (Dr D Bell, ECHA, personal communication). However, it should be noted that M8OI is one of many structurally-related ionic liquids, some of which are used more widely.

A limited number of studies have been completed with salts of M8OI, with only one study available to our knowledge in a mammalian species in vivo. In this respect, the acute toxic effects of 1-methyl-3-octylimidazolium bromide [M8IO+ Br-] has been examined in mice. The study is limited to potential adverse effects up to 24 hours after i.p. administration with the authors calculating an $LD_{50\%}$ of 35.7 mg/kg body weight[40]. Ten hours after administration, the authors report histopathological changes in the liver.

PBC is a chronic cholestatic liver disease characterised by clinical chemical markers of periportal injury (e.g. raised serum ALP), histopathological changes in the periportal region (intrahepatic bile duct loss; mixed phenotype periportal inflammation and fibrosis) and high serum titres of antibodies to antigens present on the inner mitochondrial membrane (anti-mitochondrial antibodies, AMA)[10,11]. AMA is

directed to the 2-oxoacid dehydrogenases complex family of enzymes in over 95% of PBC patients, likely through loss of tolerance to the dihydrolipoamide acetyltransferase (E2) and/or enzyme 3 binding protein components of the pyruvate dehydrogenase complex (i.e. PDC-E2 and PDC-E3BP respectively)[41,10,11]. There is likely a genetic link to likelihood of developing PBC since there is a sibling relative-risk of approx. 10[42]. A number of polymorphisms have been associated with disease risk such as HLA-DR8, with an odds ratio ranging from 2.4 to 3.3 depending on the population examined[4]. A number of other loci have been identified by GWAS as influencing disease risk, suggesting involvement of the innate and adaptive immune systems and signalling via the NF-κB, toll-like receptor and TNF pathways in PBC aetiology[5]. However, there remains an environmental element to disease prevalence (which might account in part for an apparent genetic association to disease incidence because most families inhabit the same environment). In this respect, epidemiological studies over the last few decades have suggested a link between PBC incidence and reservoir source for drinking water[6]; areas of heavy mining[7]; proximity to toxic landfill sites[8] and exposure to chemicals such as from the use of hair dyes[9]. These observations have been accompanied by a number of molecular studies which lend weight to the potential for xenobiotics to be a trigger for PBC. The first of these was the observation that the synthesis of peptides in which the lipoate moiety had been replaced with xenobiotic lipoate mimics showed immunoreactivity to PBC patient sera[43]. Rabbits immunized with 6-bromohexanoate coupled to BSA, but not BSA-immunized controls, developed AMA that was capable of inhibiting PDC-E2 enzymatic function and binding to peptide sequences not present in the xenobiotic carrier immunogen[44]. 2-Octynoic acid – used in cosmetics - was first identified as a potential xenobiotic trigger through screening 107 potential xenobiotic mimics coupled to the lysine residue of the immunodominant 15 amino acid peptide of the PDC-E2 inner lipoyl domain. PBC patient sera demonstrated high Ig reactivity against 2-octynoic acid-PDC-E2 peptide[45]. Optimal chemical structure of the xenobiotically modified epitope recognized by AMA-positive PBC sera was subsequently determined to be 2-nonynoic acid[46]. However, immunizing C57BL/6 mice with 2-octynoic acid coupled to BSA resulted in autoimmune cholangitis; increased serum

AMA; increased liver lymphoid cell numbers; increases in CD8(+) liver infiltrating cells, particularly CD8(+) T cells that co-express CD44 and an elevation in serum tumour necrosis factor-alpha and interferon-gamma levels[47]. Similar effects were also seen in a non-obese diabetic (NOD) congenic strain[48]. At the same time, it was demonstrated that recombinant lipoylation enzymes were capable of aberrantly incorporating xenobiotic lipoic acid analogues - including octanoic, hexanoic acids and the xenobiotic 6 bromohexanoic acid - into PDC-E2[27]. More recent studies have shown that AMA-positive PBC sera demonstrate reactivity to xenobiotics that have lipoic acid covalently modified at the disulfide ring, therefore without replacing the lipoic acid[49]. In both early and late-stage PBC the predominant Ig isotype to 6,8-bis(acetylthio) octanoic acid (SAc)-conjugated BSA was IgM, with titers higher with advanced stage disease[50]. However, whilst these studies show that exposure to a haptenised xenobiotic is capable of giving rise to immunological and pathological changes in animal models similar to the PBC in humans, none of the studies have exposed animals to xenobiotic alone to produce these effects.

It can be seen that M8OI bears some structural similarity to lipoic acid and, if metabolised to a carboxylic acid (in the environment and/or in the liver), has the potential to be incorporated into PDC-E2 in place of lipoic acid (**Supplementary Fig. 10b**). It could be envisaged that M8OI undergo omega hydroxylation, such as would occur via hepatic cytochrome P450 hydroxylation of fatty acids, followed by oxidation of the alcohol to a carboxylic acid[51]. An additional aspect likely important in disease pathogenesis is a route of excretion of these chemicals via the bile. Preliminary data in this manuscript indicate that a proportion of the intact toxic M8OI chemical passes into the bile and that concentrations may be significantly higher than those present in serum.

This paper identifies - for the first time - a xenobiotic present in the environment capable of being a potential xenobiotic trigger for PBC. It remains to be determined whether M8OI is a significant risk for triggering PBC, given its low production levels. However, since M8OI is structurally-related to other more widely used ionic liquids, there may be a hazard with these chemicals as regards PBC. However, the suggestion that structurally-related ionic liquids represent a risk for triggering PBC remains

hypothetical. A realistic assessment of risk may be determined through exposing experimental animals more extensively to these ionic liquids.

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FIGURE LEGENDS

Figure 1: Soil samples in close proximity to a landfill site contain biologically active levels of ERα activating chemicals.

A, Determination of MTT reduction in MCF-7 cells following 24h incubation with 0.1% v/v ethanol extracts. Results are expressed as the mean and SD of 3 separate determinations and are expressed as a percentage of 0.1% (v/v) ethanol vehicle. **B**, human ERα activation (ERE_3-pGL3promoter-Luc). MCF-7 cells were transfected with reporter constructs and 24 hours later, treated with 0.1% v/v of the ethanol extracts. After 24 hours exposure, reporter gene activities were determined as outline in the methods section with data the mean and SD of 3 separate transfections. **C**, dose-response effect for human ERα activation (ERE_3-pGL3promoter-Luc) in MCF-7 cells treated with the indicated dilution of landfill waste site soil ethanol extract, expressed as fold ethanol vehicle control. Data are the mean and SD of 3 separate determinations. **D**, human ERα activation (ERE_3-pGL3promoter-Luc). MCF-7 cells were transfected with reporter constructs and 18 hours later, pre-treated where indicated with the ERα antagonist ICI182780) or solvent vehicle control. Six hours later, cells were then treated with 0.1% (v/v) of the indicated ethanol extracts. After 24 hours exposure, reporter gene activities were determined as outlined in the methods section with data the mean and SD of 3 separate transfections. *Significantly different from solvent control (for soil extracts) or control vehicle for known chemicals ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups or #equivalent extract in the absence of ICI ($p < 0.05$) using Students T test (two tailed). **E**, human AR activation (prostate C3 RE_4-luciferase) activation by ethanol extracts. Reporter gene activities were determined as outlined in the methods section with data the mean and SD of at least 3 separate transfections. *Significantly different from solvent control (for soil extracts) or control vehicle for known chemicals ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **F**, human AR activation (prostate C3 RE_4-luciferase) antagonism by ethanol extracts after activation 500pM dihydrotestosterone (DHT). Reporter gene activities were determined as outlined

in the methods section with data the mean and SD of at least 3 separate transfections. Significantly different from #solvent control or *500pM DHT ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups.

Figure 2: Landfill site sampling site 1 and 2 PBS extracts inhibit proliferation and induce the apoptosis of the hepatic progenitor B-13 cell. **A**, ^{3}H-thymidine uptake in B-13 cells. B-13 cells were pre-treated with the indicated compounds or 1% (v/v) soil extracts for 6 hours prior to addition of ^{3}H-thymidine. Following an overnight exposure, ^{3}H-thymidine incorporation was determined. Results are the mean and SD of 6 separate determinations from the same experiment typical of at least 3 separate experiments. *Significantly different from respective solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **B**, B-13 cells were treated with the indicated environmental samples at a final concentration of 1% (v/v) for 24 hours prior to a medium change. Total number (left panel) and percentage viable cells (right panel) were then determined based on ability to exclude trypan blue. Data are the mean and SD of 3 separate treatments for the same experiment typical of at least 3 separate experiments. **C**, dose-response effect of PBS extracts on MTT reduction activity in B-13 cells determined after 24 hours exposure. Data are the mean and SD of 3 separate experiments. *Significantly different from 0% / PBS solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **D**, caspase 3/7 activity in B-13 cells treated as indicated with environmental samples at 1% (v/v) for 1-3 days or with 1µM oligomycin, 1µM rotenone or 1µM staurosporine for 24 hours. Following treatment, an ApoTox-glo triplex assay was used to determine caspase 3/7 activity. Results are expressed relative to the relevant vehicle treated cells and are the mean and SD of 3 separate determinations from the same experiment typical of at least 3 separate experiments. *Significantly different from solvent control (PBS) at the equivalent time point or DMSO vehicle control for known chemicals ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **E**, B-13 cells were treated with 1% (v/v) extracts for 2 days prior to genomic DNA isolation and analysis for nucleosomal

ladder formation (left panel). B-13 cells were co-treated with the caspase inhibitor Z-VAD-FMK at time of exposure to PBS extracts as indicated (right panel), nucleosomal ladders are from the same gel imaged and processed identically.

Figure 3: Landfill site PBS extracts inhibit mitochondrial oxidative phosphorylation in B-13 cells. **A**, B-13 cells cultured in normal media (containing 5.5mM glucose) or in media with the glucose substituted for 5.5mM galactose for at least 2 weeks prior to exposure to the indicated landfill PBS extract 2 dilution for 24 hours, followed by MTT reduction activity determination. Data are expressed relative to vehicle treated cells and are the mean and SD of 3 separate treatments from the same experiment typical of at least 3 separate experiments. Similar results were obtained with landfill PBS extract 1. *Significantly different from 0% / PBS solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **B**, timecourse of oxygen consumption rate (OCR) in B-13 cells treated with the indicated landfill extract. OCR was determined using a Seahorse XF analyser with the injections of 1% (v/v) PBS extracts, 1µM oligomycin, 1µM FCCP, 0.5µM and 0.5µM rotenone and antimycin A respectively as indicated. Readings were normalised to protein concentration and are the mean and SD of at least 4 readings from the same experiment, typical of at least 3 separate experiments. **C**, effect of 1% PBS extracts on the B-13 mitochondrial functions based on seahorse time course data. Data are the mean and SD of at least 4 readings from the same experiment, typical of at least 3 separate experiments. *Significantly different from PBS solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **D**, dose response effects of PBS extracts on B-13 mitochondrial function and extracellular acidification rate (ECAR). Data are the mean and SD of at least 4 readings from the same experiment, typical of at least 3 separate experiments. *Significantly different from 0% / PBS solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **E**, B-13 cells were treated with environmental samples at 1% (v/v) or 1µM oligomycin for 2 hours at the indicated glucose

concentration prior to determination of ATP content. Data are expressed relative to vehicle treated cells at 5.5mM glucose and are the mean and SD of 3 experiments, typical of at least 3 separate experiments. **F**, B-13 cells were treated with the indicated environmental samples at 1% (v/v) or 1μM oligomycin in culture medium containing the indicated concentration of glucose for 24 hours prior to Western blot analyses for phosphor-AMPK (AMPK phosphorylated at residue Thr172), total AMPK and β-actin.

Figure 4: Landfill site PBS extracts inhibit mitochondrial oxidative phosphorylation in primary human cholangiocytes. A, immunocytochemistry for the cholangiocyte marker cytokeratin 19 (CK-19). DAPI was used to identify cell nuclei. **B**, timecourse plot of OCR in primary cultures of human cholangiocytes treated with the indicated landfill extract. OCR was determined using a Seahorse XF analyser with the injections of 1% (v/v) PBS extracts, 1μM oligomycin, 1.5μM FCCP, 0.5μM and 0.5μM rotenone and antimycin A respectively as indicated. Readings were normalised to protein concentration and are the mean and SD of at least 4 separate determinations with cells from the same donor, typical of results from cells isolated from 3 donors. **C**, effect of 1% PBS extracts on the human cholangiocyte mitochondrial functions based on seahorse timecourse data. Data are the mean and SD of at least 4 readings from the same experiment, typical of results from 3 donors. *Significantly different from PBS solvent control ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups.

Figure 5: The chemical primarily responsible for the toxic effects associated with landfill PBS extracts 1 and 2 is M8OI.

A, HPLC chromatograms of PBS extracts from the indicated sites with the peak associated with toxicity indicated (by arrow, top panel), toxicity data not included. These data are from pooled fractions collected by preparative HPLC at the retention time associated with toxicity in landfill site PBS extract 2, and therefore partially-purified from soil PBS extracts on the basis of partition into the aqueous PBS phase and preparative HPLC. Control PBS extract 2 (middle panel) is provided as a chromatogram from a PBS

extract not displaying any toxic effects in B-13 cells and was typical of other non-toxic PBS extracts from both control and landfill sites. **B,** mass spectrometry (MS) (upper panel) and MS/MS (lower panel) for peak in panel A associated with toxicity in B-13 cells. **C**, predicted structure of peak in panel A associated with toxicity in B-13 cells, based additionally on NMR data (see **Supplementary Fig. 7a,b**).

Figure 6: M8OI recapitulates the mitochondrial effects and induces apoptosis in B-13 cells. A, timecourse of oxygen consumption rate (OCR) in B-13 cells treated with the indicated landfill extract. OCR was determined using a Seahorse XF analyser with the injections of 1% (v/v) PBS extracts, 1µM oligomycin, 1µM FCCP, 0.5µM and 0.5µM rotenone and antimycin A respectively as indicated. Readings were normalised to protein concentration and are the mean and SD of at least 4 readings from the same experiment, typical of at least 3 separate experiments. **B,** dose-response effects of M8OI on mitochondrial parameters in B-13 cells, $^{*}\rightarrow$Significantly different OCR from control vehicle at this concentration of M8OI and higher ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **C**, effect of authentic M80I (chloride salt), PBS extracts or other indicated mitochondrial toxins on ATP content in B-13 cells after 2 hours exposure. Data are the mean and SD of 3 separate treatments for the same experiment typical of at least 3 separate experiments. *Significantly different from PBS control vehicle ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **D**, effect of authentic M80I (chloride salt), PBS extracts or staurosporine on caspase 3/7 activities in B-13 cells after exposure for 2 days. Data are the mean and SD of 3 separate treatments for the same experiment typical of at least 3 separate experiments. *Significantly different from PBS control vehicle ($p < 0.05$) using ANOVA/Bonferroni-Holm comparison between groups. **E**, B-13 cells were treated with the indicated concentration of M8OI or staurosporine for the indicated periods prior to genomic DNA isolation and analysis for nucleosomal ladder formation.

Figure 7: M8OI is metabolised to COOH7IM in human hepatocytes and mice and is enzymatically incorporated into an unlipoylated fragment of PDC-E2 in vitro. **A,** LC-HR-MS/MS using a TripleTOF 5600 high-resolution quadrupole time-of-flight (TOF) mass spectrometer (Sciex) analyses of 3 separate human hepatocyte cultures incubated with M8OI for 24 hours prior to detection of M8OI, HO8IM and COOH7IM. **B**, Detection of M8OI in mouse sera orally exposed to control (left panels) or M8OI (right panels) in their drinking water as outlined in the methods section. Position of HO8IM and COOH7IM peaks is indicated by arrows; **C**, mean and standard deviation concentration of M8OI in mouse sera and gall bladder bile in control (3 mice) and M8OI exposed mice (5 mice), *Significantly different from control bile ($p < 0.05$) using the Student's T test (two tailed). **D,** Illustration of 2 step procedure for incorporation of lipoic acid into an unlipoylated fragment of PDC-E2 (ULip). LAE, lipoate activating enzyme; LT, lipoyl-AMP(GMP):N-lysine lipoyl transferase; ULip, unlipoylated fragment of PDC-E2; Lip, lipoylated fragment of PDC-E2. **E**, Western blot for the detection of ULip and Lip after addition of lipoic acid (LA) or COOH7IM, with additions as indicated.

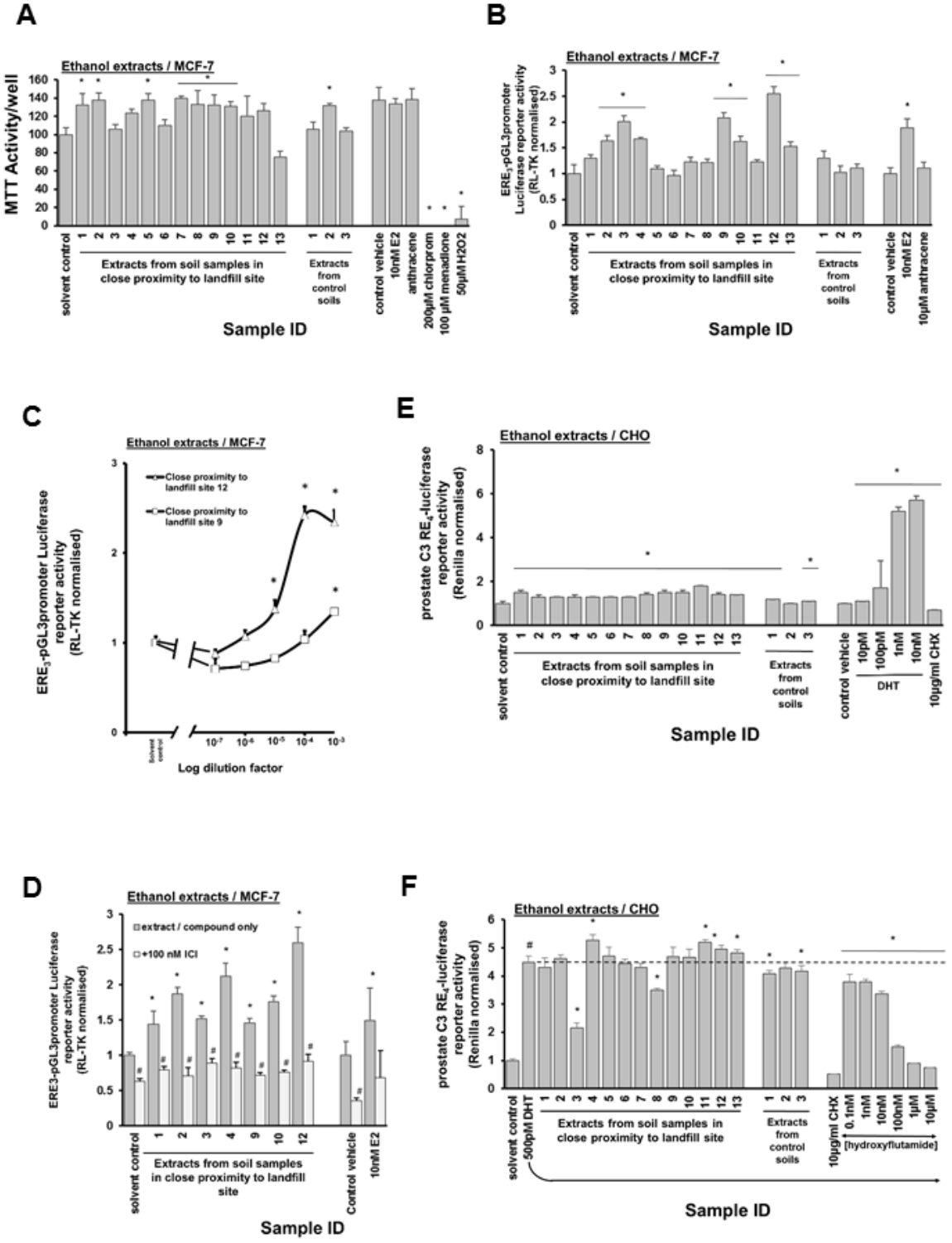

A
Ethanol extracts / MCF-7
MTT Activity/well
solvent control
Extracts from soil samples in close proximity to landfill site
Extracts from control soils
control vehicle
10nM E2
anthracene
200µM chlorprom
100 µM menadione
50µM H2O2
Sample ID
B
Ethanol extracts / MCF-7
ERE3-pGL3promoter Luciferase reporter activity (RL-TK normalised)
solvent control
Extracts from soil samples in close proximity to landfill site
Extracts from control soils
control vehicle
10nM E2
10µM anthracene
Sample ID
C
Ethanol extracts / MCF-7
Close proximity to landfill site 12
Close proximity to landfill site 9
ERE3-pGL3promoter Luciferase reporter activity (RL-TK normalised)
Log dilution factor
E
Ethanol extracts / CHO
prostate C3 RE4-luciferase reporter activity (Renilla normalised)
solvent control
Extracts from soil samples in close proximity to landfill site
Extracts from control soils
control vehicle
10pM
100pM
1nM
10nM
DHT
10µg/ml CHX
Sample ID
D
Ethanol extracts / MCF-7
extract / compound only
+100 nM ICI
ERE3-pGL3promoter Luciferase reporter activity (RL-TK normalised)
solvent control
Extracts from soil samples in close proximity to landfill site
Control vehicle
10nM E2
Sample ID
F
Ethanol extracts / CHO
prostate C3 RE4-luciferase reporter activity (Renilla normalised)
solvent control
500pM DHT
Extracts from soil samples in close proximity to landfill site
Extracts from control soils
10µg/ml CHX
0.1nM
1nM
10nM
100nM
1µM
10µM
[hydroxyflutamide]
Sample ID

Figure 1

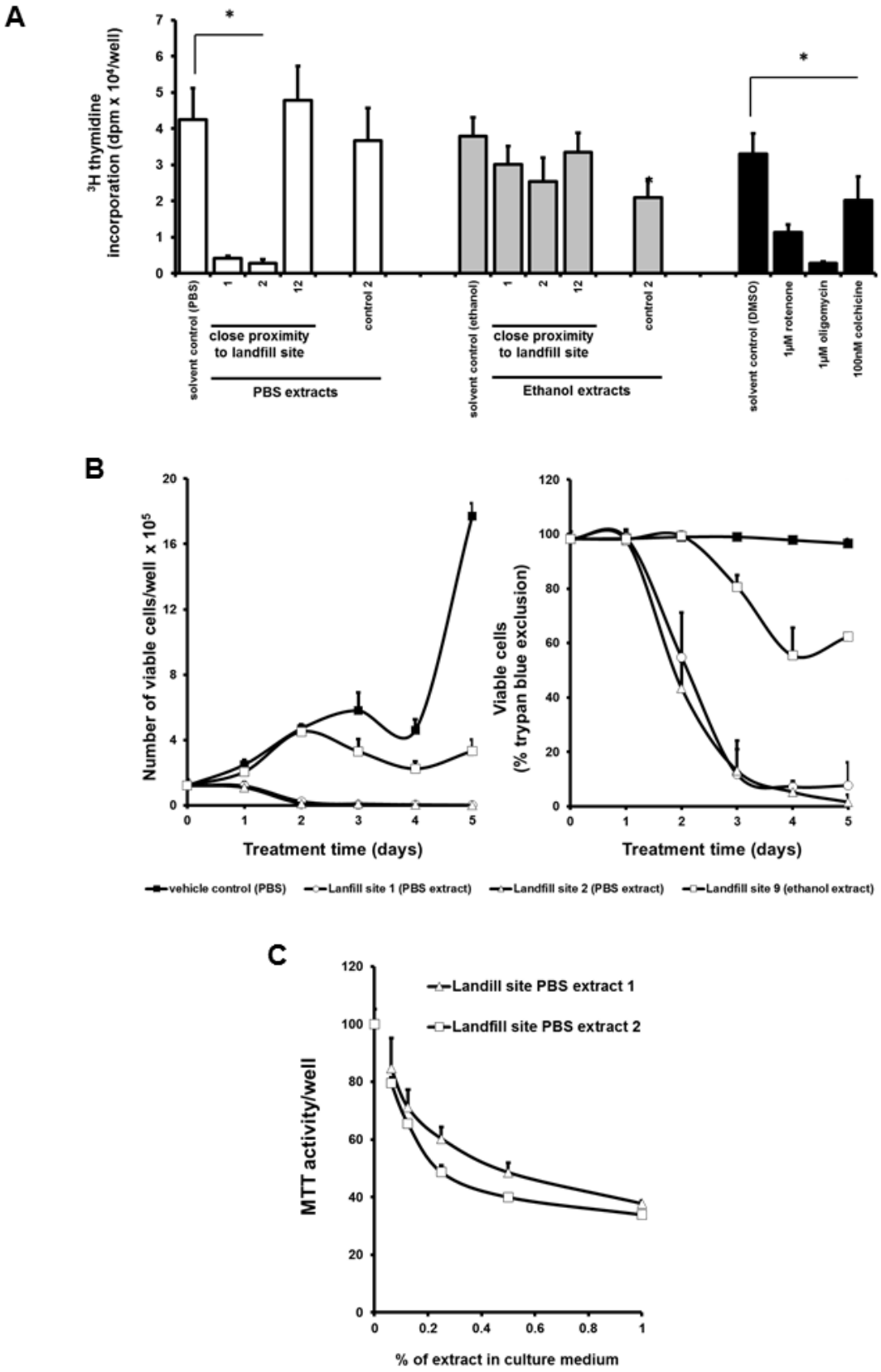

A
^{3}H thymidine incorporation (dpm x 10^4/well)
*
*
solvent control (PBS)
1
2
12
control 2
close proximity to landfill site
PBS extracts
solvent control (ethanol)
1
2
12
control 2
close proximity to landfill site
Ethanol extracts
solvent control (DMSO)
1µM rotenone
1µM oligomycin
100nM colchicine
B
Number of viable cells/well x 10^5
Treatment time (days)
Viable cells (% trypan blue exclusion)
Treatment time (days)
vehicle control (PBS)
Lanfill site 1 (PBS extract)
Landfill site 2 (PBS extract)
Landfill site 9 (ethanol extract)
C
Landill site PBS extract 1
Landfill site PBS extract 2
MTT activity/well
% of extract in culture medium

Figure 2

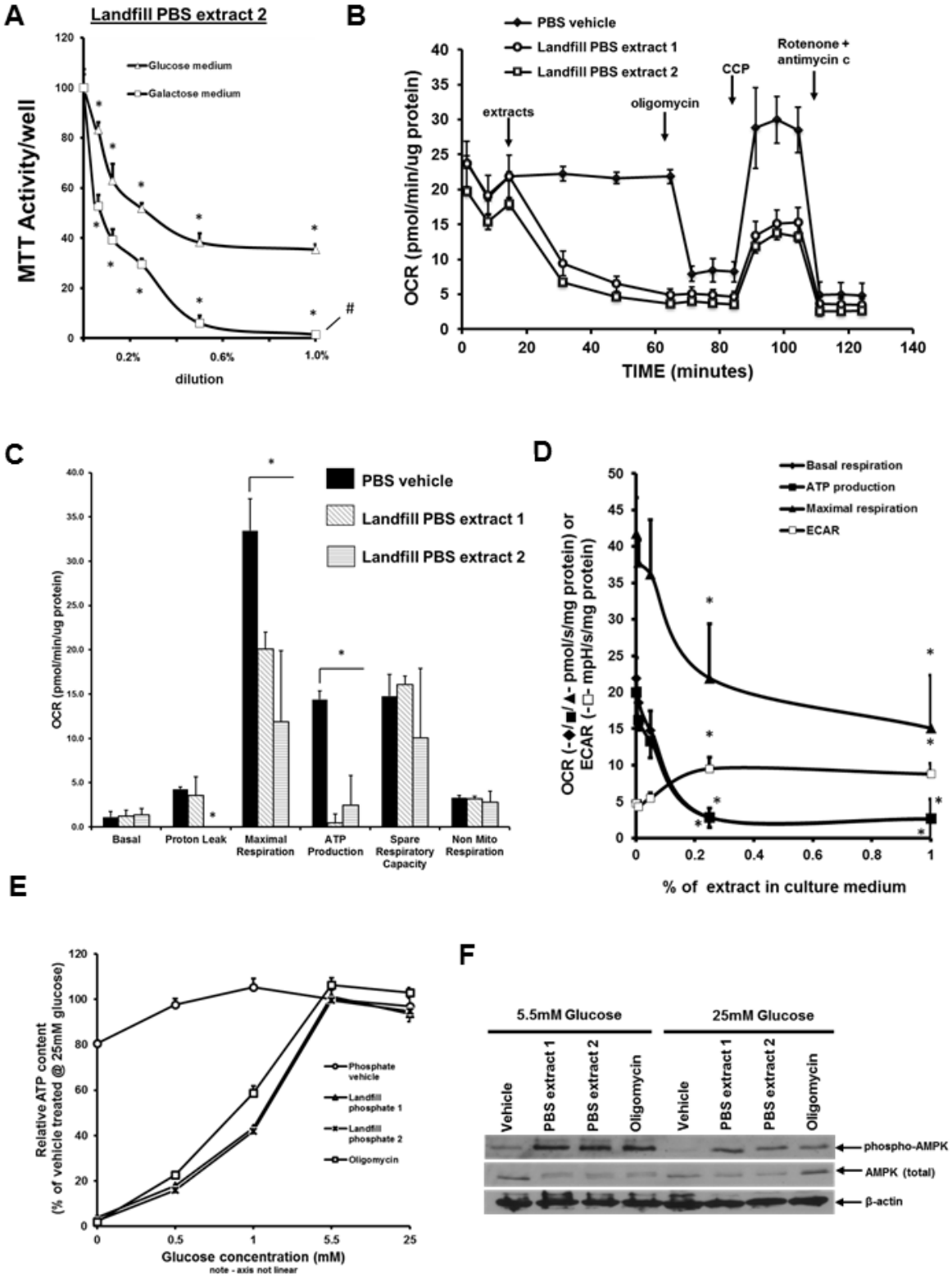
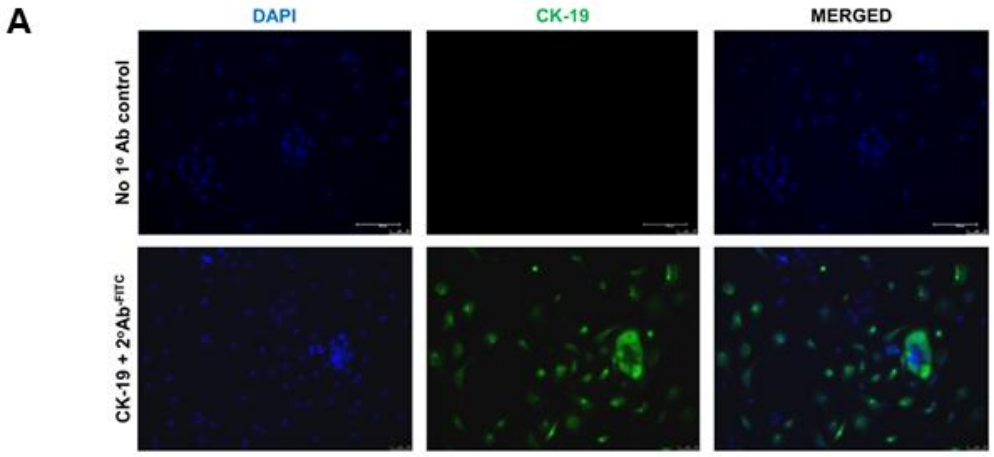
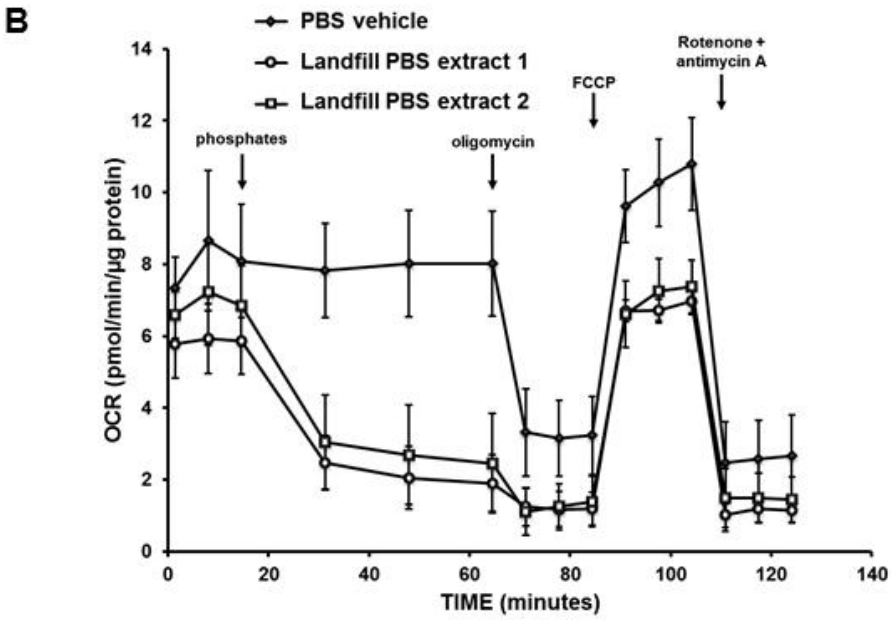

A
Landfill PBS extract 2
MTT Activity/well
Glucose medium
Galactose medium
dilution
0.2%
0.6%
1.0%
B
PBS vehicle
Landfill PBS extract 1
Landfill PBS extract 2
extracts
oligomycin
CCP
Rotenone + antimycin c
OCR (pmol/min/ug protein)
TIME (minutes)
C
PBS vehicle
Landfill PBS extract 1
Landfill PBS extract 2
OCR (pmol/min/ug protein)
Basal
Proton Leak
Maximal Respiration
ATP Production
Spare Respiratory Capacity
Non Mito Respiration
D
Basal respiration
ATP production
Maximal respiration
ECAR
OCR (-◆/■/▲- pmol/s/mg protein) or ECAR (-□- mpH/s/mg protein)
% of extract in culture medium
E
Relative ATP content (% of vehicle treated @ 25mM glucose)
Phosphate vehicle
Landfill phosphate 1
Landfill phosphate 2
Oligomycin
Glucose concentration (mM)
note - axis not linear
F
5.5mM Glucose
25mM Glucose
Vehicle
PBS extract 1
PBS extract 2
Oligomycin
phospho-AMPK
AMPK (total)
β-actin

Figure 3





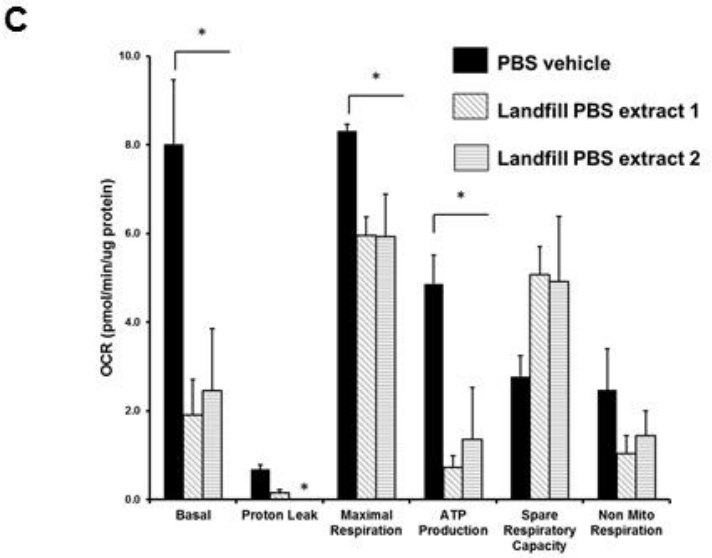


Figure 4

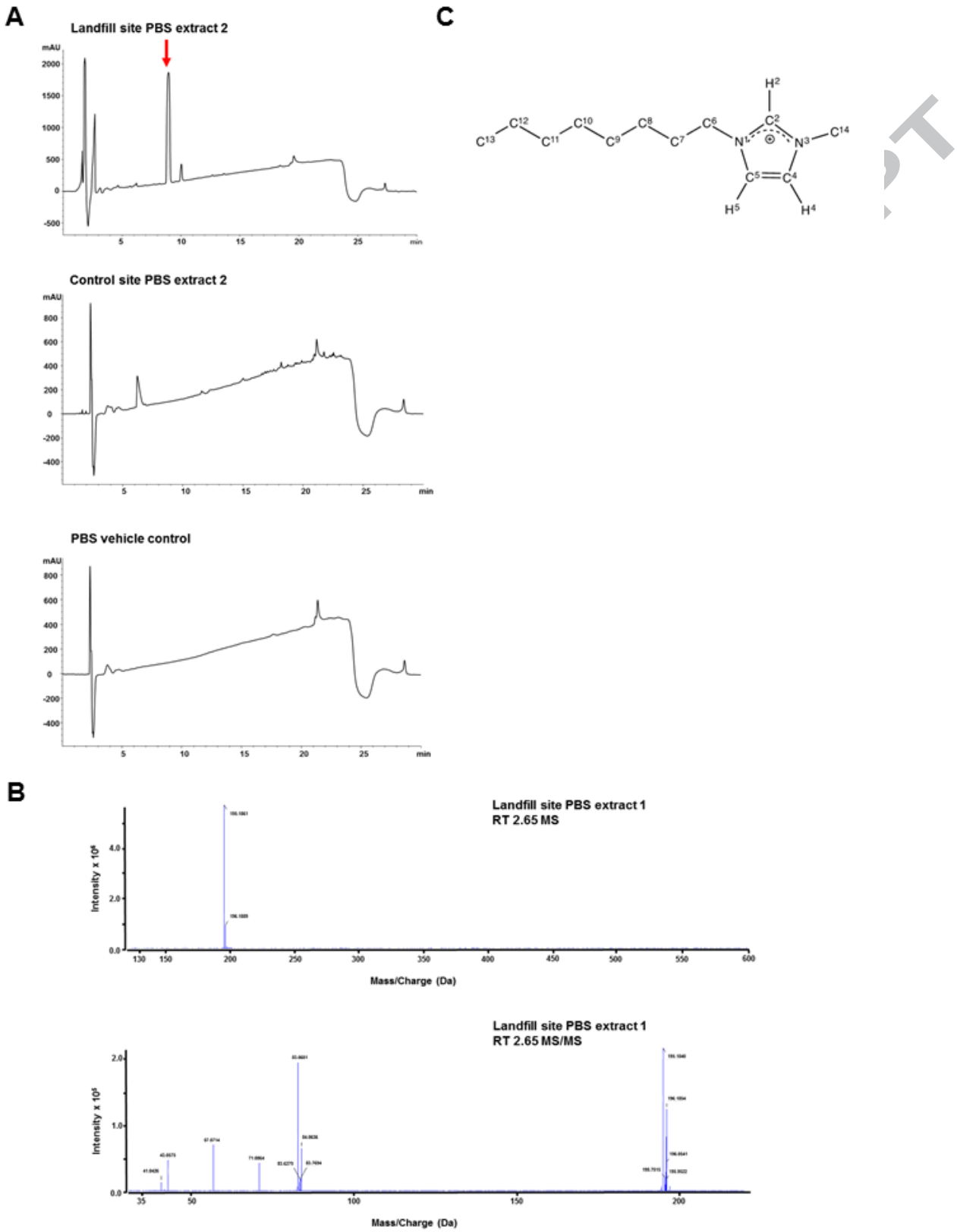

A
Landfill site PBS extract 2
mAU
min
Control site PBS extract 2
mAU
min
PBS vehicle control
mAU
min
C
B
Landfill site PBS extract 1
RT 2.65 MS
Intensity x 10⁴
Mass/Charge (Da)
Landfill site PBS extract 1
RT 2.65 MS/MS
Intensity x 10⁴
Mass/Charge (Da)

Figure 5

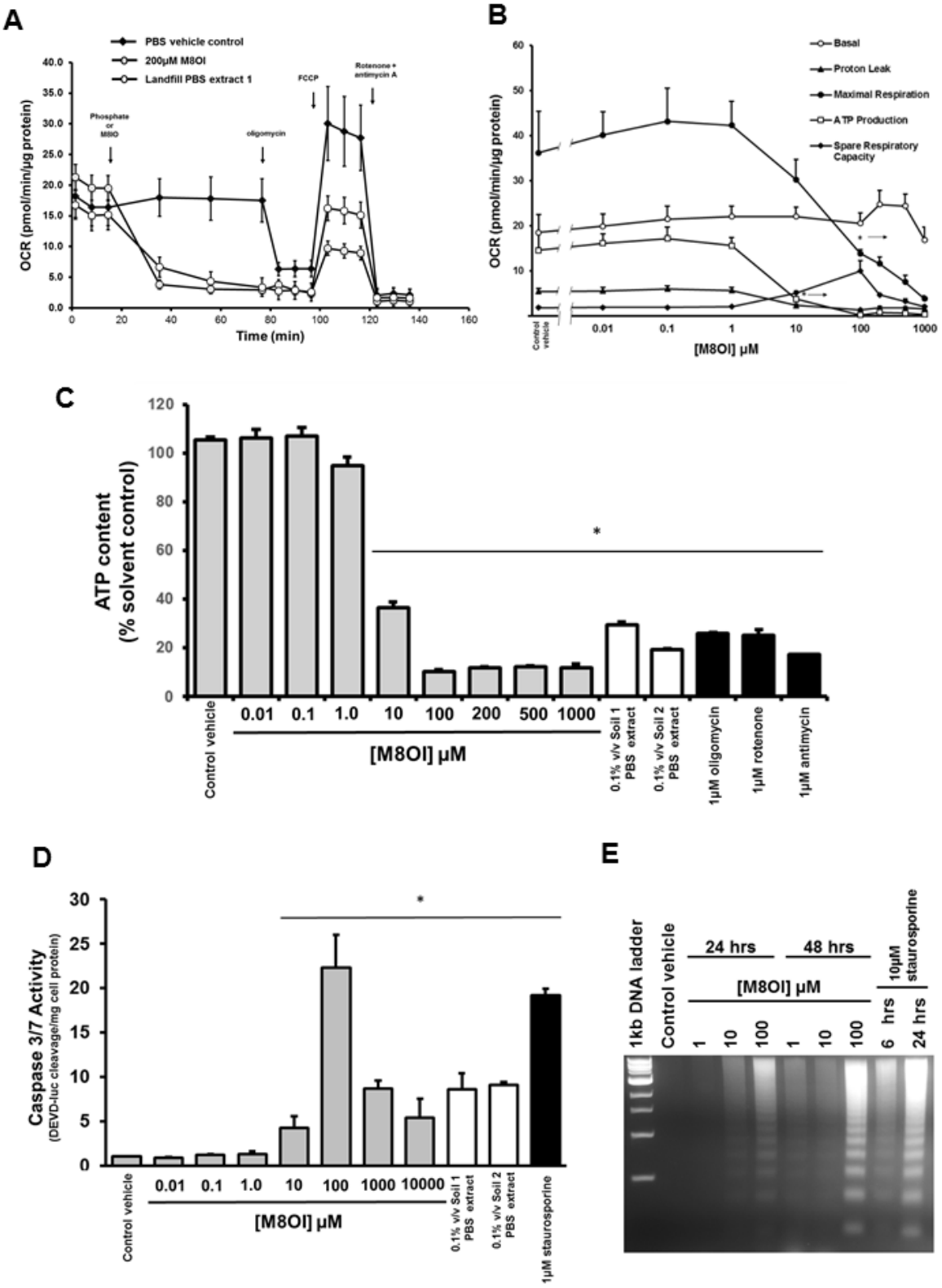

A
PBS vehicle control
200µM M8OI
Landfill PBS extract 1
Phosphate or M8IO
oligomycin
FCCP
Rotenone + antimycin A
OCR (pmol/min/µg protein)
Time (min)
B
Basal
Proton Leak
Maximal Respiration
ATP Production
Spare Respiratory Capacity
OCR (pmol/min/µg protein)
Control vehicle
[M8OI] µM
C
ATP content (% solvent control)
Control vehicle
0.01 0.1 1.0 10 100 200 500 1000
[M8OI] µM
0.1% v/v Soil 1 PBS extract
0.1% v/v Soil 2 PBS extract
1µM oligomycin
1µM rotenone
1µM antimycin
D
Caspase 3/7 Activity
(DEVD-luc cleavage/mg cell protein)
Control vehicle
0.01 0.1 1.0 10 100 1000 10000
[M8OI] µM
0.1% v/v Soil 1 PBS extract
0.1% v/v Soil 2 PBS extract
1µM staurosporine
E
1kb DNA ladder
Control vehicle
24 hrs
48 hrs
[M8OI] µM
1 10 100 1 10 100
10µM staurosporine
6 hrs
24 hrs

Figure 6

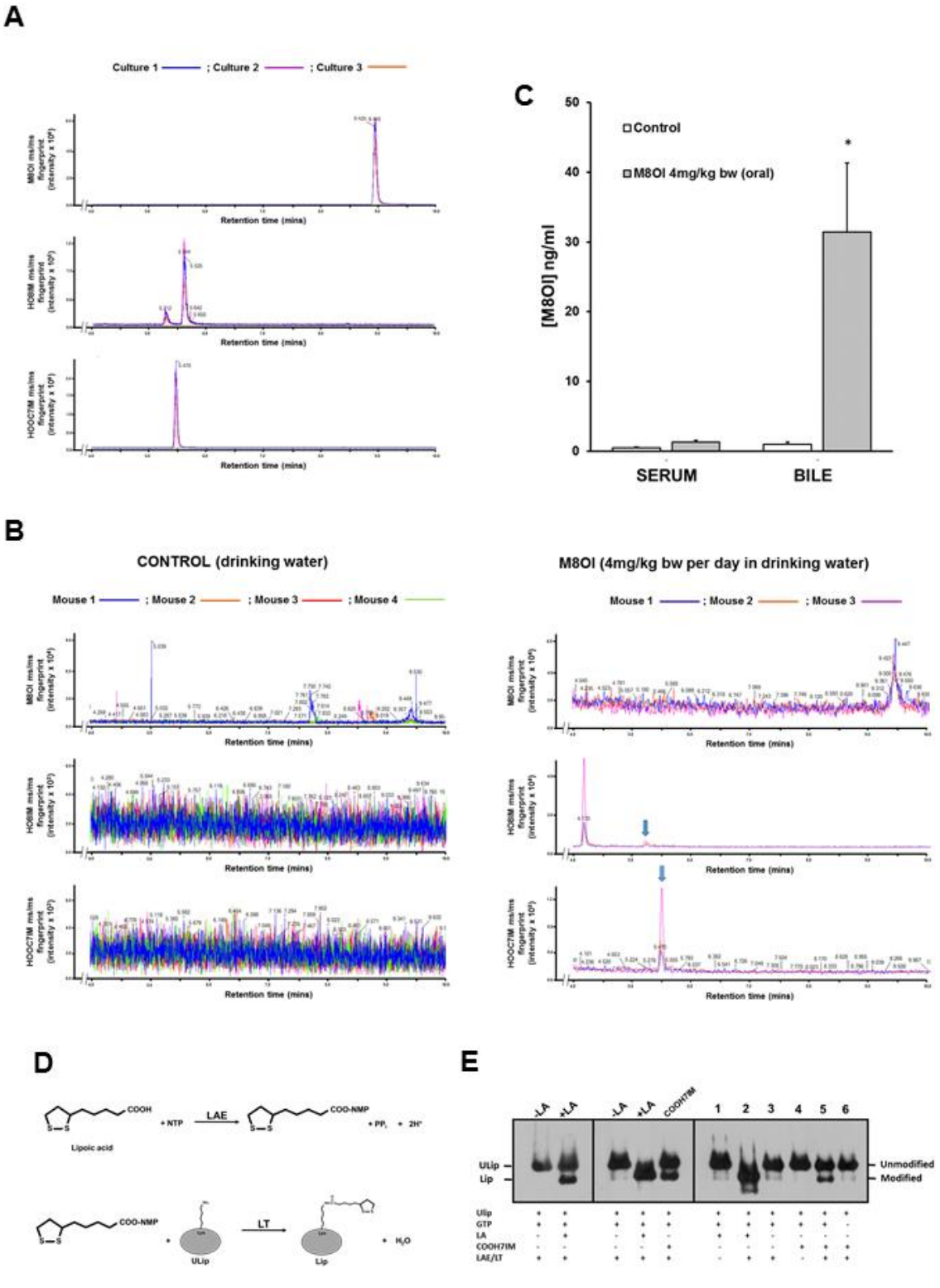

A
Culture 1 ; Culture 2 ; Culture 3
M8OI ms/ms fingerprint (intensity x 10⁴)
H08M ms/ms fingerprint (intensity x 10⁴)
HOOC7IM ms/ms fingerprint (intensity x 10⁴)
Retention time (mins)
C
Control
M8OI 4mg/kg bw (oral)
[M8OI] ng/ml
SERUM
BILE
B
CONTROL (drinking water)
Mouse 1 ; Mouse 2 ; Mouse 3 ; Mouse 4
M8OI (4mg/kg bw per day in drinking water)
Mouse 1 ; Mouse 2 ; Mouse 3
D
COOH
+ NTP
LAE
COO-NMP
+ PPi + 2H+
Lipoic acid
LT
ULip
Lip
+ H2O
E
-LA
+LA
COOH7IM
1 2 3 4 5 6
ULip
Lip
Unmodified
Modified
GTP
LA
COOH7IM
LAE/LT

Figure 7

Highlights

- Soil around a land fill waste site and control sites were analysed for heavy metals, polyaromatic hydrocarbons, pesticides and screened for their ability to activate receptors for xenobiotics and to be toxic.
- Xenoestrogens were present at higher levels in soils around a land fill site.
- An ionic liquid was found to be present at high levels in 2 soil sampling sites around the land fill.
- The ionic liquid inhibited cellular oxidative phosphorylation, was toxic to a liver progenitor cell line and induced progenitor cell apoptosis.
- The ionic liquid was also metabolised by human hepatocytes to a carboxylic acid that bore structural similarity to lipoic acid and was capable of being enzymatically incorporated into a recombinant fragment of PDC-E2 in place of lipoic acid.